

Loss of a reporter gene for green fluorescent protein during tumor progression suggests the recruitment of host cells in rats with experimentally induced colon cancer

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Summary. The interactions between a host's normal cells and tumor cells appear to be of significant importance during the development of tumors. In the present study, we examined this issue using a cancer model *in vivo* in which tumor cells were tagged with a reporter gene for green fluorescent protein (GFP). We used a model of colon cancer in immunocompetent rats, which were given a subcutaneous injection of tumor cells that had been transfected with a gene for GFP. We found that the number of fluorescent cells decreased with the progression of the primary tumors and that lymph node and lung metastases were never macroscopically fluorescent. No GFP-encoding sequences were detected by PCR in many of the long-term primary tumors, in most lymph node metastases (86%) and in all lung metastases, whereas the detection of mutated *k-ras*, which identified such cells as tumor cells, was always positive. To explain these findings, we present a brief review of the literature and postulate that tumor growth did not occur exclusively as a result of the division of the injected cells, but also involved recruitment of host cells.

Key words: Green fluorescent protein, Cancer, Metastasis, Immunocompetent rat

Introduction

Interactions between the host's normal cells and tumor cells appear to play a significant role in the development of tumors. The recruitment of host cells by tumors has been demonstrated during angiogenesis (see, for review, Hoffman, 2005), and it has been proposed that normal stem cells might be involved in the development of malignancy (Glinsky, 2005). However, many questions remain unanswered, and it is unknown whether a host's normal cells can become tumorigenic.

Green fluorescent protein (GFP) has been successfully included in many models of cancer as a marker for tumor cells. This reporter protein has many advantages; for example, no exogenously added substrate is required to visualize its expression and GFP allows the detection of single-cell metastases *in vivo* (Paris and Sesboué, 2004).

The gene for GFP has frequently been used to analyze early steps in metastasis (e.g., Mook et al., 2003, Sturm et al., 2003, Paris and Sesboué, 2004, Thews et al., 2005). However, little data about later stages have been reported. In addition, in some previous studies, metastases were not derived from the primary tumor, but were produced by intravenous or intrasplenic injection of tumor cells (Mook et al., 2003, Sturm et al., 2003), which did not allow observations of the growth of primary tumors and the subsequent spreading of metastases to distant targets, as occurs in many human cancers.

The goal of the present study was to examine the recruitment of host cells into tumors, using a model of colon cancer in immunocompetent rats, which were injected with GFP-tagged tumor cells to produce a

generalized metastatic disease with long-term follow-up.

Materials and methods

Cell culture

We used DHD/K12-PROb cells (rat colon cancer cells; also called DHD/K12-TRb cells; referred to as DHD cells herein) as malignant cells. DHD cells, which harbor a point mutation in codon 12 (GGT→GAT) of exon 1 of the *k-ras* oncogene (García-Olmo et al., 2005), were transfected with a gene for “enhanced-GFP” using retroviral supernatants from RetroFect® (retroviral particle production kit; Cellerix SL, Madrid, Spain).

Cells were cultured in monolayer in a mixture of DMEM and Ham’s F10 (1:1, v/v; Gibco-BRL, Life Technologies Ltd., Paisley, Scotland), supplemented with 10% fetal bovine serum (Gibco-BRL) and gentamycin (0.005%; Gibco-BRL). Cells were passaged after dispersion in 0.125% trypsin in EDTA.

Animals

We used both male and female BD-IX rats. They were taken from a colony established at the authors’ animal facility from founders purchased from a commercial breeder (Charles River Laboratories España, Barcelona, Spain). Breeding was performed in compliance with European Community Directive 86/609/CEE and Spanish law (Real Decreto 1201/2005) for the use of laboratory animals. As recommended by the Federation of European Laboratory Animal Science Associations (FELASA), rats in the animal facility are tested periodically to ensure that the colony is free of pathogens such as *Mycoplasma pulmonis*, *Salmonella* sp., Sendai virus, Hantaan virus and Toolan H1 virus.

From birth to the end of the experiments, all rats had unlimited access to water and standard rat chow (Panlab s.l., Barcelona, Spain). At the beginning of the experiments, rats were six to eight weeks old.

Implantation of tumors and design of experiments

Tumors were generated in the thoracic region by unilateral subcutaneous injection of GFP-transfected DHD cells into the right side of the chest. Cells were trypsinized, washed, and resuspended in phosphate-buffered saline (PBS). Then, 0.25 ml of this suspension, containing 1×10^6 cells, were injected per rat.

After injection, each rat was randomly assigned to one of three groups designated according to the time between the subcutaneous injection of cells and euthanasia, as follows: Group E, sacrificed at early stages of tumor progression (one to four weeks after injection of tumor cells; $n=6$); group M, sacrificed at middle stages (seven weeks after injection; $n=3$); and group L, sacrificed at late stages, when most of the animals had lymph node macrometastases and some also had lung macrometastases (ten to fourteen weeks after

injection; $n=11$).

An additional group of animals was included (group X; $n=2$), in which a larger number of GFP-transfected tumor cells (3.5 million) was inoculated by four subcutaneous injections. Animals in this group were sacrificed ten weeks after injection.

The growth of subcutaneous tumors was monitored in all animals and recorded weekly. We measured the greatest diameter of each tumor with electronic callipers.

Collection of tumors

On the schedule indicated above, rats were anesthetized with an intraperitoneal injection of a mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg) and a lethal dose of sodium thiopental was administered intracardially immediately afterwards.

Lungs and lymph node were inspected visually for the presence or absence of metastases, which was recorded. We also recorded the presence or absence of metastases in the peritoneum and other organs. To this end, we made two perpendicular incisions in the abdomen of each rat to allow a comprehensive inspection of the cavity upon separation of the edges of the incision.

Tumors (the primary tumor and metastases, if any) were removed, and tumor cells from individual tumors and metastases were cultured separately.

Culture of tumors

Tumor samples were macerated and then digested in a solution containing 50 mg/dL collagenase type I (Gibco-BRL) in Hank’s Balanced Salt Solution (Gibco-BRL). The digestion was carried out at 37°C for 1.5 to 2 hours. After two washes in PBS, cell suspensions were placed in sterile plates and cultured with the medium described for DHD cells, supplemented with 1% penicillin/streptomycin and 1/1000 fungizone.

PCR analyses

Each culture, after cells had been cultured for 7-14 days, was examined by PCR for the presence of the GFP-encoding sequence, the wild-type *k-ras* sequence and the mutated *k-ras* sequence.

After dispersion in 0.125% trypsin in EDTA, DNA was extracted from cells with a commercial kit (QIAamp DNA Mini Kit; QIAGEN, Hilden, Germany), according to the instructions from the manufacturer. To verify the effectiveness of the extraction, the DNA was quantified by spectrophotometry with a NanoDrop ND-1000 system (NanoDrop Technologies, Inc., Wilmington, DE, USA).

For detection of the GFP-encoding sequence, we used the following set of primers to amplify a specific 760-bp fragment: Forward, 5'-ATTGGTCTCACATGCCCGCCATGAAGATCGAG; Reverse, 5'-AATGCGGCCGCTTCAGGCGAATGCGATCGG. The reaction

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mixture, with a total volume of 50 μ l, contained a minimum of 25 ng of template DNA in a volume of 5 μ l. It also contained 0.8 μ M each primer, and 1.5 mM $MgCl_2$.

PCRs were performed with a thermal cycler (iCycler; Bio-Rad, Hercules CA, USA), with initial denaturation of the samples by incubation at 94°C for 10 min. Then amplification was allowed to proceed for 35 cycles of incubation for 1 min at 94°C, for 30 sec at 60°C and for 45 sec at 72°C, with final extension for 10 min at 72°C.

In these amplification experiments, we included DNA from GFP-transfected DHD cells, as positive control, and DNA from wild-type DHD cells and water, as negative controls.

For detection of both mutated and non-mutated *k-ras* sequences, we performed 'mutant allele-specific amplification' (MASA) by PCR, as previously described by our group (Fernández-Vega et al., 2002). Using this technique, we examined the presence of a point mutation (GGT→GAT) in the *k-ras* oncogene (exon 1, codon 12) that is found in DHD cells.

Results

After transfection of DHD cells, the percentage of cultured cells that expressed GFP was 98.6% at the beginning of the study and was never below 90.7%, as measured by flow cytometry.

In animals, tumors were detectable from the first week after inoculation of GFP-transfected DHD cells and grew at an apparently continuous rate for the duration of the experiment. The tumor diameter was 0.2 ± 0.04 cm (mean $\pm$ s.d.) one week post inoculation,

0.7 ± 0.3 cm after two weeks, 0.8 ± 0.3 cm after three, 0.9 ± 0.3 cm after four, 1.1 ± 0.4 cm after five, 1.4 ± 0.5 cm after six, 2.0 ± 0.6 cm after seven, 2.2 ± 0.8 cm after eight, 2.5 ± 0.8 cm after nine, 3.2 ± 0.9 cm after ten, 3.5 ± 0.9 cm after eleven; 3.7 ± 0.9 cm after twelve; and 3.7 ± 0.6 cm after thirteen weeks.

Tumor-bearing rats in group E did not have any visible lymph node or lung metastases. Macroscopically, green-fluorescing regions were observed in all subcutaneous primary tumors, even under conventional illumination (Fig. 1). However, non-fluorescent regions were also observed in the primary tumors from animals sacrificed two weeks after inoculation. In the cultures from the primary tumors, we always observed fluorescent and non-fluorescent tumor cells, with the former predominating, except in the cultures from animals sacrificed four weeks after inoculation, in which the proportion of fluorescent cells was smaller than that of non-fluorescent cells. PCR yielded positive results for all tested sequences in all these cultures of tumor cells (Fig. 2).

We found no metastases in tumor-bearing animals in group M. In one of these rats, no green region was evident in the primary tumor when it was removed. After culturing cells from this tumor, we failed to detect any fluorescent cells; however, we did detect the GFP-encoding sequence by PCR. In the other animals in this group, most of each tumor mass was non-fluorescent at necropsy and, similarly, green cells were in the minority in the resultant cultures. However, the GFP-encoding sequence was detected by PCR (Fig. 2). The three cultures of tumor cells in this group were all positive for both mutated and non-mutated *k-ras* sequences.

At the latest stages analyzed (group L), we observed

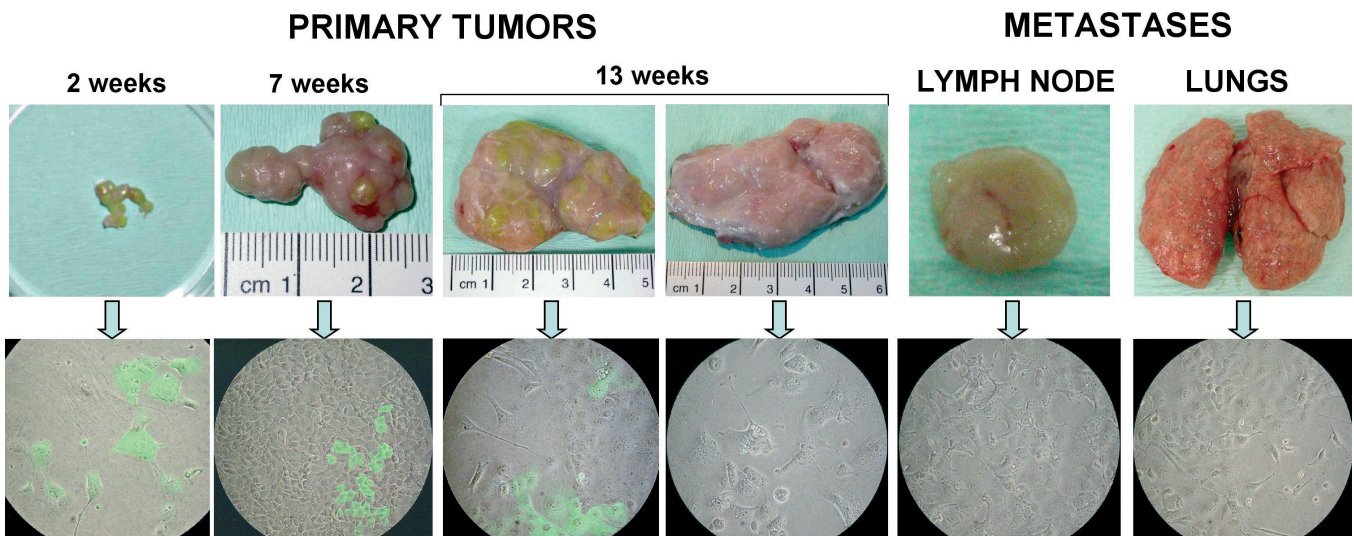


Fig. 1. Macroscopic and microscopic images of primary tumors, at different stages, and of metastases from tumor-bearing BD-IX rats. Each microscopic image was assembled by overlapping photographs that had been acquired by phase-contrast microscopy and fluorescence microscopy (blue light), respectively. $\times 40$.

green regions in the primary tumors from only three animals (27%; Fig. 1). In two other animals, cultured primary tumors were positive for the GFP-encoding sequence, in spite of the fact that we could not detect any fluorescent cells by microscopy. Thus, GFP-transfected tumor cells were still present in only 45% of the animals sacrificed at the late stages of tumor progression and, in addition, these cells were in a minority, as shown by images from cultures of tumor cells (Fig. 1). PCR for the detection of both wild-type and mutated *k-ras* sequences yielded positive results in all cases, even in those that were negative for the GFP-encoding sequence.

In six animals, we found metastases in the axillary lymph nodes. No lymph node tumors were fluorescent and in only one culture from these metastases was the GFP-encoding sequence detected by PCR. All such cultures were positive for mutated and non-mutated *k-*

ras sequences.

We found lung macrometastases in two animals and they were not macroscopically fluorescent. When we cultured the cells from these macrometastases we did not detect any fluorescence by microscopy and, in addition, PCR for detection of the GFP-encoding sequence gave a negative result, whereas both mutated and non-mutated *k-ras* sequences were detected.

In animals in group X, tumors grew at a constant rate in all injection sites and we found lymph node and lung macrometastases when the rats were sacrificed, ten weeks after inoculation. In only one rat, the primary tumor had green regions at necropsy and the GFP-encoding sequence was detected. No metastases were macroscopically fluorescent in either of the two animals and the GFP-encoding sequence was not detected in cultures of cells from lymph node and lung metastases.

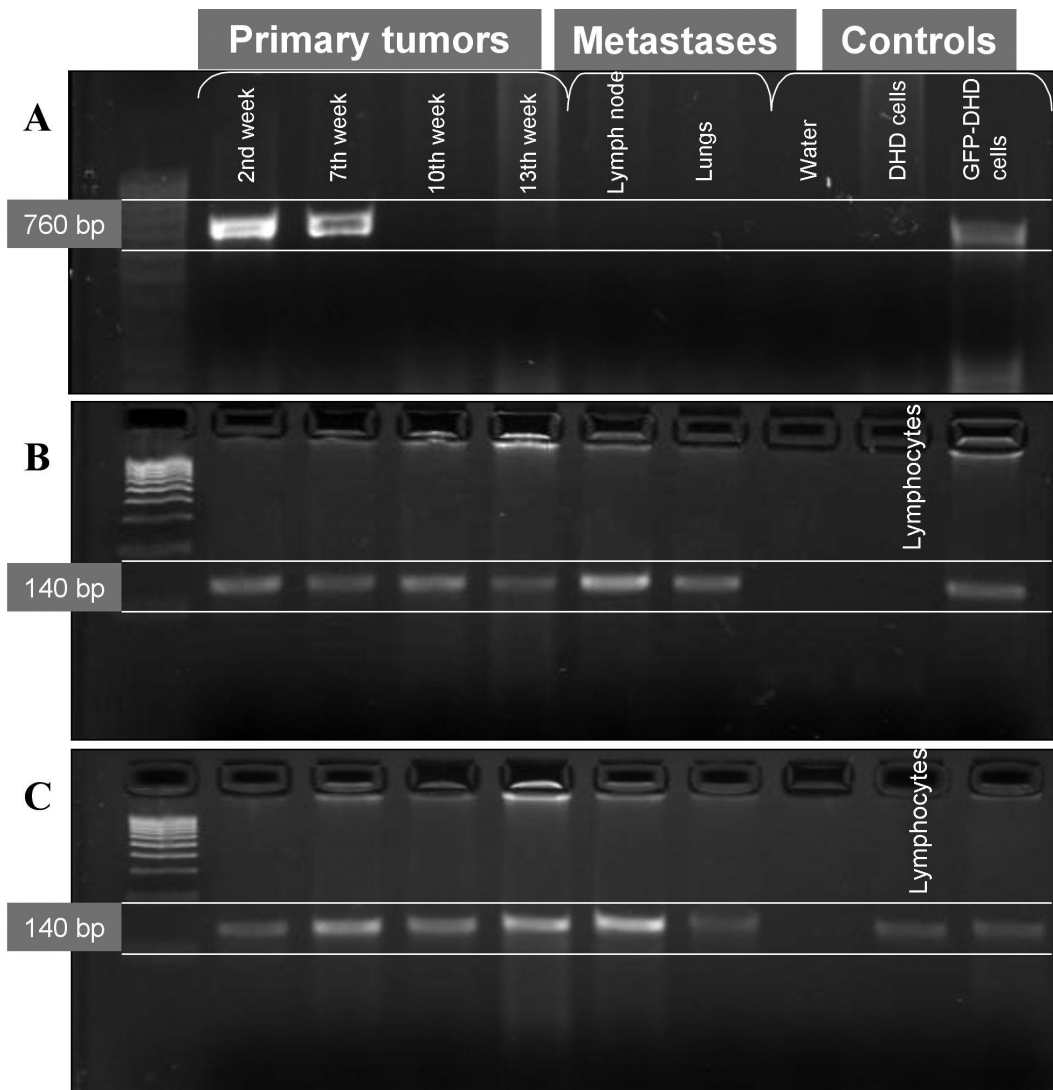


Fig. 2. Photographs of three agarose gels, showing the results of analysis by PCR for the detection of the GFP-encoding sequence (A), the mutated *k-ras* sequence (B) and the non-mutated *k-ras* sequence (C) in cultures derived from rat primary tumors and metastases. Lane 1: Molecular mass markers. Lanes 2-5: Amplification of DNA extracted from primary tumors obtained from four rats that were sacrificed at different times after inoculation of GFP-transfected DHD cells. Lanes 6-7: Amplification of DNA extracted from metastases. Lanes 8-10: Controls for PCR, namely, water (no template); DNA from wild-type DHD cells as template (negative control for the detection of the GFP-encoding sequence); DNA from lymphocytes from healthy rats as template (negative control for the detection of the mutated *k-ras* sequence); and DNA from GFP-transfected DHD cells as template (positive control for the three amplifications by PCR). Bp: Base pairs.

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All such cultures were positive for mutated and non-mutated *k-ras* sequences.

An assay of the sensitivity of the PCR technique for detection of the GFP-encoding sequence revealed that we were able to detect one GFP-transfected DHD cell in more than 10 million wild-type DHD cells.

Discussion

In the present study, we used a syngenic rat model of colon cancer, in which tumors were produced in immunocompetent BD-IX rats by inoculation of rat colon adenocarcinoma cells that had been tagged with a reporter gene for GFP. This model allowed us to analyze the growth and spread of tumors in a setting closer to clinical disease than is possible with the use of immunocompromised animals, since host reactions might be essential to the phenomena that we hoped to examine.

It has been reported that the long-term development of metastases generated from GFP-transfected tumor cells is markedly inhibited in immunocompetent animals, and it has been suggested that use of GFP-labeled tumor cells in such animals should be limited to tracking the early stages of tumor metastasis exclusively (Steinbauer et al., 2003). In fact, we observed that the growth of primary tumors and the development of macrometastases in rats injected with GFP-transfected DHD cells were slightly slower than in other animals injected in the same way with wild-type DHD cells (data not shown). To examine this issue, we injected a larger number of GFP-transfected DHD cells (3.5 million) into two rats. Their tumors grew at a constant rate and we found lymph node and lung macrometastases when the rats were sacrificed, as little as ten weeks after inoculation. Thus, it appears that the immune response to GFP is not able to fully inhibit the development of metastases, and that models in immunocompetent animals should not be discarded in studies of long-term metastases.

Most previous studies of metastasis using fluorescent proteins have been based on the detection of micrometastases by direct observations of fluorescence (Mook et al., 2003, Yamamoto et al., 2003, Steinbauer et al., 2003). However, it has been reported that the fluorescence of GFP decreases with reduced oxygenation, which is a characteristic of both experimental and clinical tumors (Coralli et al., 2001). Thus, it was theoretically possible that cells did not express GFP, even if they harbored the sequence that encodes this protein. To circumvent this problem, we examined the presence of the GFP-encoding sequence by PCR in all cultures established from animal tumors. In addition, the detection of the mutated *k-ras* sequence provided proof that cultured cells were tumor cells. We cultured tumor cells before analyses by PCR, since, in many tumors, there were non-fluorescent regions or mixed green-fluorescing and non-fluorescing regions. In this way, we avoided false results related to sampling.

We found that primary tumors contained both fluorescent and non-fluorescent tumor cells when animals were sacrificed between the second and the fourth week after the inoculation of GFP-transfected tumor cells. At later stages, tumors did not always contain fluorescent regions and the GFP-encoding sequence could not even be detected in many of them. However, the cultured cells derived from the tumors still harbored the mutated *k-ras* sequence, which identified them as tumor cells. Lymph node and lung metastases were never macroscopically fluorescent and the GFP-encoding sequence was undetectable in most of such lymph node metastases (86%) and in all lung metastases, whereas mutated *k-ras* was always detected.

With respect to the detection of the GFP marker protein in the two animals injected with a larger number of cells, the results were in full agreement with those obtained in the previous groups: the primary tumor had green regions at necropsy in only one rat; no metastases were macroscopically fluorescent in either of the two animals; and the GFP-encoding sequence was detected in only one primary tumor, but not in cultures of cells from lymph node and lung metastases.

These findings are hard to explain in terms of the instability of transfection, since the GFP-transfected DHD cells had been maintained *in vitro*, without selection pressure, for more than thirteen months (more than 100 passages) with no significant loss of fluorescent cells, as determined by flow cytometry. Specifically, at the beginning of this study, the frequency of fluorescent cells was 98.6% and 13.5 months later it was 90.7%. In addition, a previous study using a similar method for transfection of tumor cells, demonstrated the adequate stability of the fluorescence *in vitro*, as well as *in vivo*, when GFP-transfected cells were inoculated into athymic mice and monitored for 3-6 months (Goodison et al., 2003). Also, it has been reported that GFP-transfected rat sarcoma cells expressed GFP after at least 19 passages *in vivo* and, later, after subcutaneous injection into immunocompetent rats (Thews et al., 2005).

Our results indicate not only the loss of expression of the fluorescent protein, but also the loss of the GFP-encoding sequence. To our knowledge, no previous study has shown that the GFP reporter gene can disappear *in vivo* during tumor progression. But, as far as we know, the present study is the first in which GFP-transfected tumor cells have been inoculated into immunocompetent animals to produce a generalized metastatic disease with long-term follow-up.

The apparently inexplicable loss of the reporter gene with tumor progression led us to postulate that tumor growth did not occur only via division of the injected cells, but also via recruitment of host cells. This hypothesis would explain why, at middle and late stages of tumor progression, both GFP-positive (inoculated cells) and GFP-negative (possible host-derived) tumor cells were found in primary tumors. Moreover, the frequency of GFP-negative cells in the primary cultures

from tumor cells increased with tumor progression.

Our results for metastases are consistent with the putative recruitment of host cells, since the marker gene for GFP was not found in any distant metastases. This result led us to postulate that such metastases had developed exclusively from transformed host cells.

Spontaneous fusion *in vivo* between host cells and tumor cells and its possible contribution to tumor progression and metastasis were suggested more than two decades ago (Kerbel et al., 1983). In the cited study, such host cells were not identified, but the authors suggested the bone-marrow origin of at least some of them (Kerbel et al., 1983). A few years later, Lagarde suggested that host cells of lymphoreticular origin were most likely involved in the process (Lagarde, 1986).

Currently, the hypothesis that stem cells might be recruited into tumors and that this phenomenon might contribute to the tumor's microenvironment, growth and progression is gathering strength, and is supported by a variety of previous observations (Glinsky, 2005). In fact, integration of the results obtained by many different research groups is close to giving a clearer picture of the possible roles of stem cells in cancer, with stem cells, rather than somatic cells, being considered as the origin of cancer cells (for review, see García-Olmo et al., 2004).

We did not identify the host cells that might have been involved in tumor progression in our rat model. However, in preliminary experiments, we cultured fetal rat cells with supernatants from cultures of GFP-transfected DHD cells. Three to five days later, we found, as expected, fluorescent tumor cells and non-fluorescent fetal cells, but we also found non-fluorescent tumor-like cells and fluorescent fetal-like cells. Thus, it appeared that there was an interaction between the fetal cells and the GFP-transfected tumor cells (or material released from them) that resulted in an interchange of genes. While these experiments were only exploratory, it appeared that pluripotent progenitor cells were able to acquire phenotypic characteristics of tumor cells when co-cultured with them.

At the molecular level, the recruitment of host cells into tumors might be explained by mechanisms that have been shown to be operative *in vivo*, such as spontaneous fusion (Kerbel et al., 1983) or the spontaneous uptake of biologically active genetic material, which has been demonstrated in mammalian cells (Lehmann and Sczakiel, 2005; Stroun and Anker, 2005).

Some years ago, our group proposed that cell-free DNA derived from tumors might be biologically active, and that distant metastases might occur by transfection of stem cells with cell-free nucleic acids from tumors (García-Olmo et al., 2000). The phenomenon proposed by this "hypothesis of genomestasis" is consistent with the findings in our present study and especially with the differences found between the tumor cells injected into rats and the metastatic cells found some weeks later. However, in light of our present results, this hypothesis might be even expandable to include the growth of the

primary tumor, since it appeared that host cells were also recruited to the primary tumor.

In conclusion, the results of the present and previous studies suggest a new concept to explain cancer progression that integrates host stem cells and their recruitment to tumors.

Acknowledgements. The authors thank Dr. Antonio Bernad and Dr. Daniel Rubio for their invaluable advice during the course of this study. This work was financed in part by grants from Fondo Europeo de Desarrollo Regional and Fondo de Investigación Sanitaria (no. PI031540) and Junta de Comunidades de Castilla La Mancha (no. PI03047), Spain. J. Samos and M.G. Picazo were recipients of fellowships from Junta de Comunidades de Castilla-La Mancha, Spain (nos. JI04011 and GC03010, respectively).

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Accepted May 13, 2008